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Multivariate Analysis of the Effects of Manganese on the Reproductive Physiology and Behavior of the Male House Mouse

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MULTIVARIATE ANALYSIS OF THE EFFECTS OF MANGANESE ON THE REPRODUCTIVE PHYSIOLOGY AND BEHAVIOR OF THE MALE HOUSE MOUSE

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Chronic exposure to Mn_3O_4 in the diet at 1050 ppm Mn retarded the sexual development and lowered reactive locomotor activity levels in male mice. A multivariate analysis of variance indicated that testis, seminal vesicle, and preputial gland weights were significantly smaller as a result of Mn administration. These results support earlier observations of altered locomotor activity levels and reproductive development in male rats in the absence of other signs of toxicity.

INTRODUCTION

Manganese is used in a variety of industrial processes including steelmaking and the manufacture of dry-cell batteries. It has been added to fuel oils as a smoke inhibitor and as a substitute for Pb in gasoline, and it is released into the atmosphere as a result of the utilization of coal. These processes release tens of thousands of metric tons of Mn oxides into the atmosphere each year (U.S. Environmental Protection Agency, 1975). Although Mn is ubiquitous in the atmosphere because of these uses, the biological effects of long-term, low-level exposure to these compounds are generally unknown. It is known that chronic occupational exposure to high levels of Mn dust or fumes from mining and metallurgical operations occasionally causes a progressive deterioration of the central nervous system and induces subtle behavioral changes in less severe cases (Cotzias, 1958).

Although several investigators have reported that Mn exposure produces tremors, ataxia, and other severe neurological disorders in animals, an appropriate animal model of the less severe cases of Mn poisoning has not been described. Recently, however, studies in our laboratory have indicated that chronic dietary exposure to Mn (Mn_3O_4) retards reproductive development (J. W. Laskey, G. L. Rehnberg, J. F. Hein, S. C. Carter, in preparation) and alters reactive locomotor activity (RLA) in the rat

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(Gray et al., 1978) in the absence of any obvious clinical signs of manganism. The present study was designed to determine the reproducibility of these effects in another rodent species, the house mouse. We expected that Mn administration would reduce locomotor activity and retard reproductive development, with no effects on growth of non-reproductive tissues and no gross signs of neurotoxicity in male mice.

In this study we used multivariate statistics to describe and analyze the results, because multiple indices of reproductive development were obtained for each animal, including testis, seminal vesicle, and preputial gland weights. Repeated utilization of univariate analyses of variance (ANOVAs) in such a situation results in a high likelihood of type I and type II errors, which in turn allows data interpretation to become very subjective (Harris, 1975). A two-way multivariate ANOVA was used to test the hypotheses that age and Mn treatment alter reproductive development. It was anticipated that the growth of all three tissues would be retarded by Mn treatment, as their growth is dependent in part on testosterone.

GENERAL METHODS

Pregnant mice, *Mus musculus* (CD-1), shipped on d 12 of gestation from Charles River Breeding Laboratories (Wilmington, Mass.), were housed individually in transparent plastic mouse cages (33 X 20 X 13 cm). All animals were maintained on a 12:12 light:dark photoperiod with the lights going out at 6 p.m. EST. These dams were placed on a casein-based diet (Table 1) on d 13 of gestation, and on d 15 of lactation 26 healthy litters were randomly assigned to one of two treatment regimens. The control group was continued on the casein diet containing 50 ppm Mn as $MnSO_4$; the diet of the treated group contained an additional 1050 ppm Mn as Mn_3O_4. This dose level was selected because it was effective in previous studies with rats (J. W. Laskey, in preparation). At 30 d of age the males were weaned and housed in groups of two or three with littermates. For the duration of the experiment, the mice were given fresh chow three times a week and their growth, general appearance, and the presence of any gross behavioral abnormalities (i.e., tremors, ataxia, hind limb paralysis) were noted.

SUBJECTS

At 58 d of age, four nonsibling males from each treatment group were killed and weighed and the wet weights of their paired preputial glands, seminal vesicles, and paired testes were recorded. The sex accessory glands were measured because they depend on testosterone for growth (Hafez, 1970). At 73 d of age, 15 control males and 11 males from the Mn-treated litters were tested during the day for 2-h RLA levels in a

TABLE 1. Dietary Components of the Teklab (Madison, Wisc.) Casein Diet

Component	Formula	Amount (g/kg)
Casein, high protein		210.0
DI-Methionine		1.0
Sucrose		576.2298
Corn starch		100.0
Corn oil		50.0
Vitamin mix, Teklab (catalog no. 40060)		10.0
Calcium carbonate, AR[a]	$CaCO_3$	20.0
Sodium phosphate, monobasic, AR	$NaH_2PO_4 \cdot H_2O$	20.0
Potassium chloride, AR	KCl	5.0
Sodium chloride		5.0
Magnesium sulfate, AR	$MgSO_4$	1.993
Zinc sulfate, AR	$ZnSO_4 \cdot 7H_2O$	0.5307
Manganese sulfate, AR	$MnSO_4 \cdot H_2O$	0.1548
Cupric sulfate, AR	$CuSO_4 \cdot 5H_2O$	0.0198
Potassium iodate, AR	KIO_3	0.0017
Ferrous ammonium sulfate, AR	$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.1404

[a] AR, analytical reagent.

residential maze (Reiter et al., 1977). Each maze contains eight photocells, and as an animal ambulates, photocell beam interruptions are recorded. The total number of beam interruptions occurring in a 2-h period is a measure of locomotor activity. After behavioral testing, 17 control and 12 treated males were killed and their preputial, seminal vesicle, testis, and body weights were recorded. At 90 d of age, 22 control and 10 Mn-treated males were similarly necropsied. In addition, the liver and kidney weights were taken in a few cases at 73 and 90 d.

STATISTICAL METHODS

A univariate, one-way ANOVA was calculated by a regression procedure to assess the effect of Mn on RLA levels. A two-way multivariate analysis of variance (MANOVA) (Barr et al., 1976) was then used to determine whether reproductive development, which included preputial, testis, and seminal vesicle weights, was affected by Mn administration or age. From this analysis a new composite variable, the discriminant function, was derived. This new variable is a linear weighted sum of these three original variables, and a t-test of the significance of the treatment effect is identical to the results of the MANOVA. The discriminant functions (L) were calculated for each treatment group from the equation $L = a_1$ (seminal vesicle weight) + a_2 (testis weight) + a_3 (preputial gland weight) (Li, 1964). The weighting factors (a_1, a_2, and a_3) were calculated so that the linear combination of the three values maximally discriminates between the control and Mn groups. The weighting factors were obtained

by multiplying the inverse of the variance-covariance matrix ($\mathbf{S}^{-1}$) by a difference matrix (d) containing the difference between the mean control and Mn values for the three parameters. The differences between the means were calculated from the partial sums of squares due to treatment for each variable and are therefore adjusted for age. The treatment means are adjusted for age because sample sizes are not equal at each age and having more controls at the greater age inflates the sequential sums of squares due to treatment.

It is important to note that MANOVA has certain assumptions similar to those of the univariate analyses, including multivariate normality and homogeneity of the covariance matrices between treatments. Although tests for multivariate normality are unavailable, sufficiently large samples have a multivariate normal distribution (Harris, 1975). On the other hand, when there are large discrepancies in sample sizes, as in the present study, heterogeneity of the covariance matrices can lead to large discrepancies between the true and reported significance levels. Therefore a χ^2 test of homogeneity between covariance matrices was performed (Harris, 1975).

RESULTS

The growth and general appearance of the Mn-treated animals was normal throughout the experiment. Administration of 1050 ppm Mn did not induce tremors, ataxia, or hind limb paralysis, which are characteristic of severe cases of manganism in humans. However, the activity levels of the Mn-treated males were significantly reduced (Fig. 1) at 73 d of age.

Growth of the reproductive tissues was retarded by Mn administration [$F(3,63) = 6.05$, $p < 0.002$ as analyzed by a MANOVA] (Table 2). The matrix (d) calculated from the partial sums of squares due to Mn treatment for each variable was $d' = (31.3, 29.36, 24.65)$, the values of d' being the differences between the treatment means for each parameter adjusted for age for the seminal vesicles, testes, and preputials, respectively. Multiplication of ($\mathbf{S}^{-1}$) by (d) produced weighting factors that have been incorporated into a linear equation for the new variable, $L = 0.0064052$ (seminal vesicle weight) + 0.0205147 (testis weight) + 0.0226385 (preputial weight). The values of the discriminant functions were 9.2454 ± 0.16 and 8.0906 ± 0.21 for the control and Mn groups. A Student's t-test of this statistic yields $T^2 = 18.72$, and $F = [(N-P-1)/(N-2)p]\,T^2$, with $N = 69$ and p (variates) = 3, and therefore $F = 6.05$.

The χ^2 test indicated that the covariance matrices were homogeneous ($\chi^2 = 5.5$ with 6 d.f., not significant) with respect to treatment. The MANOVA also indicated that these reproductive tissues were growing from 54 to 90 d of age [$F(6.124) = 10.6$, $p < 0.0001$].

Unlike reproductive development, body, liver, and kidney weights were unaffected by treatment (Table 2).

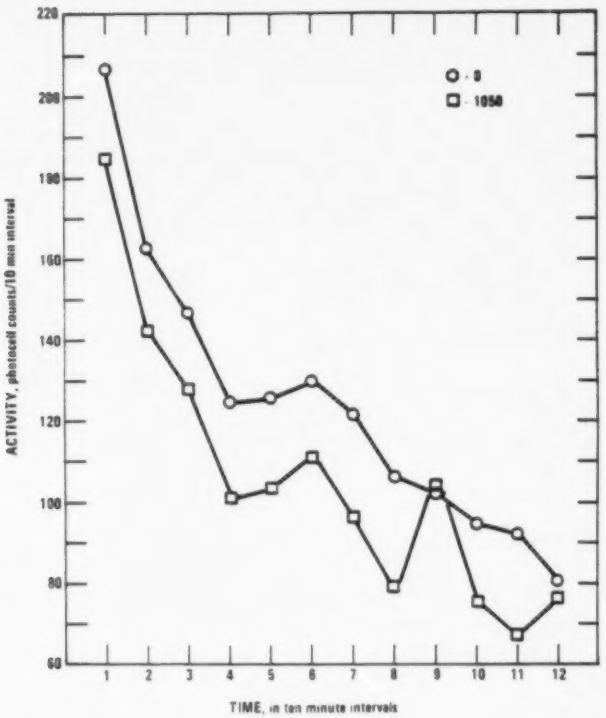

FIGURE 1. Effects of Mn_3O_4 (1050 ppm Mn) on the 2-h RLA levels of 70-d-old male mice. Total activities for control and Mn-treated mice were 1504 ± 40 and 1336 ± 49 photocell counts/2 h, respectively [$F(1,24) = 7.14, p < 0.015$].

DISCUSSION

Results of the present study demonstrate that chronic dietary administration of Mn_3O_4 to the male mouse alters behavioral and reproductive development. These results are consistent with our previous findings that Mn treatment produced hypoactivity and retarded reproductive development in rats. However, the reproductive development of the mouse seems to be more susceptible than that of the rat to the inhibitory effects of Mn. In the male rat, J. W. Laskey et al. (in preparation) observed a developmental alteration of serum pituitary and testicular hormone levels, but did not see a reduction in testicular growth even at Mn levels that were considerably higher (3500 ppm) than those used in the present study. Rodier (1955) reported that 81% of his patients with Mn poisoning had reduced levels of urinary 17-ketosteroids, the urinary

TABLE 2. Effects of Chronic Dietary Administration of Mn_3O_4 on Growth and Reproductive Development of Male Mice at 58, 73, and 90 d of Age

Measurement	Age (d)		
	58	73	90
Body weight (g)			
Control	28.8 ± 1.8[a]	29.7 ± 0.6	34.6 ± 0.5
Mn[b]	26.9 ± 1.0	29.9 ± 1.1	34.2 ± 0.6
Paired testes weight (mg)			
Control	195 ± 16	223 ± 5	246 ± 5
Mn	156 ± 13	208 ± 7	227 ± 13
Seminal vesicle weight (mg)			
Control	151 ± 15	209 ± 13	285 ± 14
Mn	146 ± 20	199 ± 17	223 ± 28
Preputial gland weight (mg)			
Control	106 ± 12	123 ± 7	153 ± 8
Mn	67 ± 13	109 ± 6	121 ± 9
Liver weight (mg)			
Control	–	1,732 ± 100	1,773 ± 92
Mn	–	1,807 ± 142	1,776 ± 280
Kidney weight (mg)			
Control	–	219 ± 7	284 ± 22
Mn	–	225 ± 30	331 ± 60

[a] Values are means ± SE.
[b] Mn concentration = 1050 ppm.

metabolites of androgens, which suggests that Mn may alter androgen production in humans as well as in rodents.

It is not clear from the present study whether the reduction in RLA is causally related to or only correlated with reproductive dysfunction, as both are affected by Mn treatment. In our studies with male rats, the reduction in activity occurred only after puberty (Gray et al., 1978), which suggests a causal relationship between testosterone production and reduced activity. However, rats receiving a microinjection of Mn in the caudate nucleus become lethargic (Inoue et al., 1975), raising the possibility that the reduced locomotor activity seen in the present study resulted directly from the action of Mn on the brain rather than being mediated through a reduction in gonadal hormones.

Although growth of the testes and sex accessory glands was retarded by Mn administration, body and liver weights were unaffected; this indicates a specific effect on the hypothalamic-pituitary-gonadal axis rather than a general retardation of the growth of all organs. The mechanism by which Mn affects the reproductive system is unclear and there are many plausible hypotheses. Growth of the testes is primarily the result of proliferation of the germinal epithelium, which is dependent on the gonadotrophic hormones (GnH) from the anterior pituitary and testo-

sterone from the Leydig cells. Altered GnH or testosterone synthesis or secretion during puberty could produce the reduction in weight of the testes and sex accessory gland seen in the present study. However, it is also possible that Mn may affect reproductive development at the hypothalamic level rather than the anterior pituitary. The factor (GnRH) that regulates the release of follicle stimulating hormone and luteinizing hormone from the anterior pituitary is produced in the anterior hypothalamus, and interference with GnRH release would also alter reproductive development. A single injection of anti-GnRH in the young male rat permanently retards testicular development (Bercu et al., 1977).

In the present study, we used a multivariate analysis to evaluate the effect of Mn on the reproductive tissues. Testis, seminal vesicle, and preputial gland weights are indices of reproductive development, and all three are at least partially dependent on testosterone for growth. This analysis is appropriate when multiple observations are made on every animal, as in the present study and in most toxicological experiments. We consider multivariate tests particularly useful in the interpretation of large masses of data including interrelated variables, and we recommend that other investigators consider incorporating these analyses into their experiments.

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